ORIGINAL ARTICLE

The effect of the pseudostem extract of ambon banana on the number of lymphocytes and fibroblasts in gingivitis: an experimental study

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ABSTRACT

Introduction: Gingivitis is an inflammation of the soft tissue that supports the teeth. Chemical treatment in the form of mouthwash, such as chlorhexidine, is often recommended. However, long-term use of chlorhexidine mouthwash will cause side effects, such as allergies, irritation of the oral cavity, and discoloration of the oral mucosa and teeth. Therefore, natural alternative materials containing flavonoids, saponins, and tannins that can cure gingivitis are needed. These ingredients are found in the pseudo stem of the Ambon banana. This research aims to analyze the effect of applying the pseudostem extract of Ambon banana on the number of lymphocytes and fibroblasts in treating gingivitis. **Methods**: This study was an in vivo laboratory experimental study with a post-test control group. The study used 45 Sprague Dawley rats which were divided into 3 groups, namely the positive control group (chlorhexidine gel), negative control (gel base), and treatment (Ambon Banana pseudostem extract gel). The gingiva on the mandibular incisor of the rat was ligated, then the gel was applied to the gingiva. Decapitation of rats was carried out on days 1, 3, 5, 7, and 9, and then made histological preparations with HE. Staining and observations were later made. Data analyzed using Kruskal-Wallis test. **Results**: The results showed that Ambon banana false stem extract gel reduced the number of lymphocyte cells with a significant difference of $p=0.016$ ($p<0.05$) and increased fibroblasts at $p=0.85$ ($p>0.05$). **Conclusion**: The pseudostem extract gel of Ambon banana reduced the number of lymphocyte cells and increased fibroblasts in gingivitis treatment.

KEYWORDS
fibroblasts, gingivitis, lymphocytes, pseudostem of ambon banana

INTRODUCTION

Gingivitis is a periodontal disease that affects the teeth's supporting soft tissues. The World Health Organization (WHO) reports that periodontal disease is one of the most common oral diseases in the world.[1] Periodontal disease is a condition that affects both adults and teenagers. Gingivitis and periodontitis are two common periodontal diseases in society. According to Riskesdas 2018, gingivitis is exceptionally common in Indonesia, with a prevalence of 74%.[2]

Gingivitis is caused by two factors, primary factors and secondary factors. The primary factor causing inflammation of the periodontal tissues is plaque bacteria.[3] Bacteria suspected as the cause of periodontal disease include *Aggregatibacter actinomycetemcomitans*.[4] Meanwhile, secondary factors are divided into two, local and systemic factors. Local factors include caries, failed dental restorations, orthodontic appliances, irregular tooth position, improper use of dentures, and accumulation of food debris. Systemic factors include nutritional factors, hormonal factors, hematology, psychological disorders and the use of drugs. When the gingiva is inflamed, the body's defenses are activated, and inflammatory cells such as lymphocytes are produced. Lymphocytes help the body's immune system destroy microorganisms. On the third day after the onset of inflammation, lymphocytes begin to accumulate. The increase in lymphocytes must be kept to a minimum to avoid long-term inflammation that can cause wound-healing complications.[5]

In gingivitis, tissue damage is detected not only in the epithelial cells but also in the surrounding matrix. Therefore, restoring the epithelial mass and its normal structural framework is necessary. This process begins with the formation of new blood vessels, called angiogenesis.[6] The formation of new blood vessels is considered important in healing as it supplies oxygen and nutrients to injured tissue and ensures cell-mediated and humoral immune system delivery to help prevent infection.[6,7] The activation and proliferation of fibroblasts and related mesenchymal cells (myofibroblasts) is then regulated by a number of growth factors, including platelet-derived growth factor (PDGF), tumor growth

factor/transforming growth factor-β (TGF-β), interleukin-1 (IL-1), platelet-derived growth factors (FGFs), and tumor necrosis factor (TNF-α). TGF-β is the most important pro-fibrogenic peptide. TGF-β has a primary function as an inhibitor of cell proliferation, but in granulation tissue, its main function is to activate fibroblasts to produce extracellular matrix proteins, enter the lesion, and deposit connective tissue proteins in the debrided area about 6 days after fibroblasts enter the lesion. Fibroblasts remain quiescent or inactive except during wound healing, inflammation, and cancer.[7]

The processes of angiogenesis and fibroblast proliferation lead to the development of granulation tissue.[6] A tissue can be called granulation tissue if the visible appearance of the wound on the skin surface resembles red granules and is undergoing a process that occurs in healing like most tissues. Some fibroblasts forming granulation tissue contain myofibrils and express the cytoskeletal protein - smooth muscle actin called myofibroblasts. Myofibroblasts have contractile properties and are considered to play a role in wound closure. In normal healing, contractions can be seen 6 to 12 days after injury and move inward from the wound margin at about 0.5 mm/day. The connective tissue response continues with the deposition of extracellular matrix proteins, particularly collagen and ends with matrix remodeling that includes gradual changes in the relative abundances of different matrix proteins. This process begins a few weeks after the injury and is completely completed within 2 years.[8]

Gingivitis treatment can be carried out by eliminating the main factor through plaque control. Plaque control can be done chemically or mechanically. Mechanical treatment can be conducted with scaling, while chemical treatment can be carried out with antibiotic therapy or mouthwash. The combination of scaling and the use of antibiotics has been shown to reduce the number of bacteria and the depth of pocket in the gingiva.[9,] The mouthwash that is often used is chlorhexidine. Chlorhexidine has side effects if used for a long time.[10] Another alternative that can minimize the side effects of chemical drugs is herbal medicine. Herbal medicines have very low side effects compared to chemical drugs and have pharmacological properties, so the World Health Organization (WHO) recommends using natural ingredients as herbal medicines.[10,11] One plant that has the potential of herbal medicine is the Ambon banana tree.[11]

The Ambon banana plant has many benefits the fruit can be consumed, the leaves can be used as traditional food wrappers, and the sap and stem can be used as herbal medicine.[11] Banana stems contain active compounds, namely alkaloids, flavonoids, tannins, and saponins.[12] Alkaloid compounds, saponins, tannins, flavonoids, and anthraquinones are natural compounds that have the potential as antibacterial agent.[13] Many studies have proven that Ambon banana stems have significant antibacterial potential against gram-positive bacterium, namely *Staphylococcus aureus*. It can also significantly inhibit the growth of *Streptococcus mutans, Escherichia coli, Propionibacterium acnes,* and *Candida albicans*.[14] This research aimed to analyze the effect of the pseudostem extract of Ambon banana on the number of lymphocytes and fibroblasts in treating gingivitis.

METHODS

This study was an *in vivo* laboratory experiment with a post-test control group design. This research was conducted at the Laboratory of Molecular Medicine and Therapy Research, University of Muhammadiyah Yogyakarta (Lab-MMT). The sample was obtained using the Federer formula calculation.[15] Forty-five rats were grouped into 3: positive control, negative control, and treatment group. Inclusion criteria included healthy male Sprague Dawley rats, 2-3 months old and weighing 200-250 grams. Exclusion criteria included being sick or dead rats within a certain period. Controlled variables included Sprague Dawley rats, age, weight, gender, food and drink of rats, and gel dose, while uncontrolled variables included pH conditions, saliva volume, microorganisms in the oral cavity of rats, other bacterial contamination, systemic conditions, and resistance of rats.

Ambon banana pseudostem extract was made using the Soxlethasi method with methanol as a solvent. The making of Ambon banana pseudostem extract began with washing the Ambon banana pseudostem that will be used, and then draining it. The false stems were then finely sliced, then dried and ground into powder, then filtered. The powder was weighed at 100 grams, wrapped in filter paper around each of the contents, and put into a Soxhlet tube. After that, 1000 ml of methanol solvent was put into a round-bottom flask and heated on a stove at 70 °C. The extraction process was stopped when the methanol solvent in the extraction tube looked clear (approximately 4 hours). After that, the extracted liquid was concentrated using a rotary evaporator to obtain Ambon banana pseudostem.[16]

The gel base was made by heating distilled water to a temperature of 50°C, mixing it with 0.75 g CMC-Na, 2.25 ml propylene glycol, and 4.5 ml glycerin, then stirring until a gel forms. The gel base is put into a container and then stored in the refrigerator at a temperature of 4-6°C. Ambon banana pseudostem extract gel 20% was made by dissolving 0.2 grams of Ambon banana pseudostem extract in 1 mL of distilled water, then mixing with the gel base and stirring until homogeneous. 20% of Ambon banana pseudostem extract gel was put into a container and then stored in the refrigerator at a temperature of 4-6°C.[16]

The treatment of rats by means of 45 Sprague Dawley rats was put into 3 groups containing 15 rats, namely the positive control group (Chlorhexidine gel/CHX), the negative control group (gel base), and the treatment group (Ambon banana pseudostem extract gel/ BPS). The three groups each contained five rats. Rats from each group were ligated to induce gingivitis, a 3 mm silk ligature tied to the gingival margin of the mandibular incisors for 7 days to produce gingivitis, with the appearance of the gingiva becoming red and swollen.[17] The gel preparation material was applied to the gingival sulcus based on each group. The application is done once a day, with one swipe by microbrush. Decapitation was carried out on each group of rats on days 1, 3, 5, 7, and 9. Three rats were taken from each group.

Histopathological examination was carried out by gingival tissue, which was taken to a minimum but represented the entire gingival structure. Preparations were made with hematoxylin and eosin staining.

The removed gingival tissue was fixed using a 10% formalin buffer. Gingival tissue was cut with a thickness of <1 cm, and then the tissue was inserted into a tissue cassette. Dehydration was carried out using 70%, 80%, 95%, and 100% alcohol, then the preparations were put into the xylol solutions I and II for clarification. The tissue was grown using paraffin and thawed at a temperature of 57–59 °C. Paraffin was put into the mold with half the volume and left to solidify. The frozen paraffin was then filled again to the brim in the mold. The paraffin was put in the refrigerator until it was really hard and came off the mold. The released paraffin was sliced using a microtome with a thickness of 3–4 m, then put into a water bath at 50°C. It was later removed using a glass plate.

It was incubated on a hot plate at a temperature of 40–50 °C for 15 minutes to remove the water content in the tissue. The preparations were stained using hematoxylin and eosin (HE). They were put in xylol I, II, and III for 3 minutes from each xylol, then the preparations were put in 70%, 80%, 95%, and 100% alcohol for 2 minutes from each alcohol, after which the preparations were poured with water. The preparations were put in a Mayer solution of hematoxylin for 7 minutes and then drained with running water. The preparations were put in eosin for 30 seconds, rinsed with running water, and then dehydrated by dipping each preparation three times in 70%, 80%, 95%, and 100% alcohol.

The preparations were put into a solution of xylols I and II. Preparations were stained with one drop of mounting medium and covered with a glass deck.[18] Observation and counting of preparations under an Olympus BX41 light microscope (Tokyo,Japan) with 400x magnification with 3x field of view in 3 repeated calculations connected to a computer screen and counting the number of lymphocyte cells in three fields of view.

Observation and counting of preparations under an Olympus BX41 light microscope (Tokyo, Japan) with 400x magnification and a 3x field of view in 3 repeated calculations connected to a computer screen and counting the number of lymphocyte cells in three fields of view. The mean and standard deviation was used to express the data. The Shapiro-Wilk test ($p<0.05$) was used to evaluate the normality of the data. The Kruskal-Wallis test ($p<0.05$) was used to determine the significant difference.

RESULTS

The results of the average number of lymphocyte cells from 3 viewpoints are as follows:

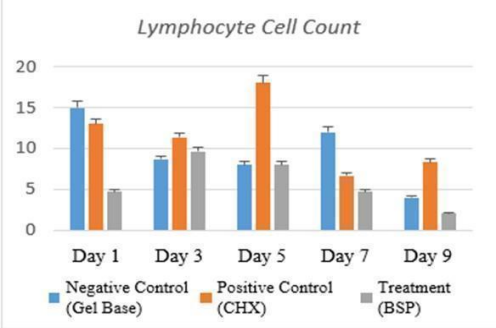

Figure 1. The decreasing pattern of the number of lymphocyte cells between the positive control (CHX), negative control (Gel Base), and treatment (BSP) groups on days 1, 3, 5, 7, and 9

Based on Figure 1, it can be seen that the average number of lymphocyte cells on day 1, 3, 5, 7, and 9 in the positive control group had the highest value than the negative control group and treatment group on day 5 with a value of 18. The treatment group had a lower mean lymphocyte cell on day 9 than the positive control and negative control groups. The variance analyzed using the Kruskal–Wallis test showed significant differences $p=0.016$ ($p <0.05$) in the average number of lymphocyte cells on days 1, 3, 5, 7, and 9.

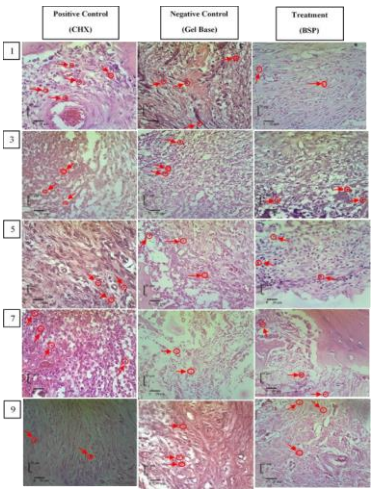

Figure 2. Histological preparation of Lymphocyte cells on days 1, 3, 5, 7, and 9, with CHX, Gel Base, and BSP staging. HE staining, 400x magnification.

The Results of histological examination of lymphocyte cells on days 1, 3, 5, 7, and 9 in the CHX (positive control), basic gel (negative control), and BPS (treatment) groups on Day 1 show that the positive control group and negative control group experienced an increase in the number of lymphocytes compared to the treatment (BPS) group, and days 5 to 9 in the BPS group showed a decrease in the number of lymphocytes compared to the CHX and base gel groups. (Figure 2).

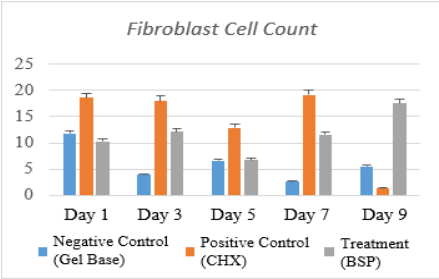

Figure 3. The increasing pattern of the number of fibroblast cells between the positive control (CHX), negative control (Gel Base), and treatment (BSP) groups on days 1, 3, 5, 7, and 9

Figure 3 showed that the number of fibroblast cells on day 1 of the positive and negative control groups was higher than that of the treatment group (BPS), but on day 9, the number of fibroblast cells in the treatment group was higher than that of the positive or negative control groups. The number of fibroblast cells in the treatment group increased, although based on analysis of variance using the Kruskal-Wallis test, it showed that there was no significant difference of $p=0.85$ ($p > 0.05$) in the average number of lymphocyte cells on days 1, 3, 5, 7, and 9.

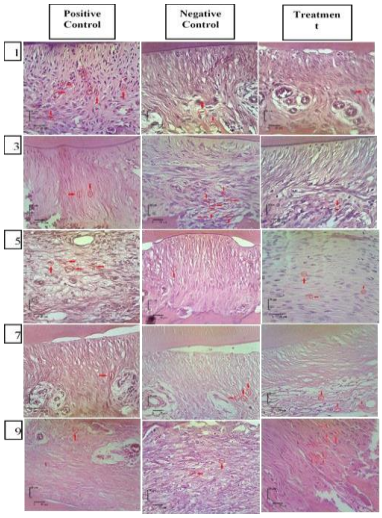

Figure 4. Histological preparation of fibroblast cells in days 1, 3, 5, 7 and 9, with CHX, Gel Base, and BSP staging. HE staining, 400x magnification.

Results of histological examination of fibroblast cells on days 1, 3, 5, 7, and 9 between CHX, base gel, and BPS, although analysis of variance using the Kruskal-Wallis test showed that there was no significant difference, p= 0.85 (p>0.05), on day 5, the number of fibroblast cells was seen to increase compared to the base gel (negative control). There was an increase in the number of fibroblast cells in the BPS group, especially on days 7 and 9 (Figure 4).

DISCUSSION

The calculation of the average lymphocyte based on figure 1 and figure 2 on day 1 showed that the positive control group and the negative control group experienced an increase in the average number of lymphocytes compared to the treatment group. According to previous research results, it was stated that lymphocyte cells would increase on day 1. The increase in lymphocytes on day 1 indicated the beginning of the inflammatory process.[19] The positive control group had been applied with chlorhexidine gel which is bacteriostatic and bactericidal, thereby adding an antibacterial agent improved the inflammatory process.[9] The negative control group also experienced an increase in the number of lymphocyte cells because the implementation of gel base of glycerin, which would increase the number of lymphocytes during the inflammatory process even though no drug was applied.[20]

On the third day, the positive control group and the negative control group experienced a decrease in the number of lymphocyte cells. It was a sign that inflammation started to be controlled. The results of wound healing research show that the number of lymphocytes increases in chronic inflammation because lymphocytes migrate to the wound area on day 1, then the amount will peak on days 3 to 6, and on the seventh day, the lymphocytes decrease. The increase in the number of lymphocyte cells on the third day occurred in the treatment group. This happens, probably because of the antigen. There were more areas of injury in this group, so the inflammatory process still occurred in the gingiva.[5,9] On the fifth day, the negative control group experienced increased lymphocyte cells due to re-inflammation. On the following day, the positive control group and the treatment group experienced a decrease in lymphocyte cells, indicating controlled inflammation. It can be characterized by a decrease in the number of inflammatory cells.[13]

On the seventh day, the average number of lymphocyte cells decreased in the negative control and treatment groups, indicating that wound healing began.[1] The number of lymphocytes decreased, indicating that the antigen was no longer present. The average number of lymphocytes in the positive control group increased due to re-inflammation, which occurred due to bacterial contamination at the time of the study. Reinfection that occurs can increase the number of lymphocytes. On the ninth day, the average number of lymphocyte cells of the negative control group increased, indicating that the re-inflammatory process had occurred due to bacterial contamination during the research. The number of lymphocyte cells in the positive control and treatment groups decreased drastically, indicating that the inflammation ended. The end of the inflammatory phase indicated that the gingivitis had healed in mice.[13]

The number of lymphocyte cells in the treatment group was always lower than the positive control group and the negative control group, except on the third day, and then decreased on the fifth day. This is in accordance with the research results of Fauzia et al., who found that lymphocytes on the third day were higher than on the fifth day.[19] It proved that the treatment group applied with the Ambon banana pseudostem extract gel was more influential in reducing the number of lymphocyte cells than the group applied with chlorhexidine gel and gel base. The pseudostem extract gel of Ambon banana has antibacterial properties such as tannins, saponins, and flavonoids to suppress the number of bacteria in the inflammatory process.[11]

The decrease in the number of lymphocyte cells in this study could be influenced by experimental animals and the manufacture of test materials. The condition of the experimental animals affected the decrease in the number of lymphocyte cells, including the body weight of rats. The researchers did not weigh the weight of the Sprague-Dawley rats at the end. It could be an indicator of whether the rats experienced stress or not. Stress in rats can reduce appetite so there is a decrease in nutrition. Nutrition also has an important role in wound healing.[7] Thus, it also affects the healing of gingivitis. The wound-healing process can also be influenced by the immune system of each experimental animal. Furthermore, the manufacture of test materials can also reduce the number of lymphocyte cells. The error that occurred when making the gel base as the material applied to the negative control group was using glycerin as raw material. Glycerin is bacteriostatic, inhibiting bacterial growth and accelerating the wound-healing process.

The positive control group was tested by applying 0.2% chlorhexidine gel. Chlorhexidine has an effect as an effective antibacterial against gram-negative, gram-positive, fungi, and yeast cells. Chlorhexidine can reduce plaque accumulation and inflammation in the oral mucosa.[9] Chlorhexidine has the ability as bactericidal and bacteriostatic. These effects can be seen from different concentrations. Chlorhexidine acts as a bacteriostatic at a low concentration of 0.1 g/ml, capable of preventing bacteria from replicating. Chlorhexidine acts as a bactericide with high concentrations (>100 g/ml), which can increase the permeability of bacterial cells by changing the protein structure of the bacterial cell membrane to inhibit bacterial growth.[3]

The negative control group was applied to a gel-based material on experimental animals. The gel base material applied contained glycerin in its composition. Glycerin has a function as a bacteriostatic and humectant.[20] Thus, the gel base suppressed gingivitis's bacterial growth which gave many benefits to the wound for excellent healing outcomes in this study.

The treatment group applied with the Ambon banana pseudostem extract gel experienced a faster inflammatory healing process than the other groups. Fauziah et al.,[19] research used lime peel extract, which contains flavonoids and saponins, to reduce lymphocytes on days three and five and accelerate

wound healing. This is in line with this research, as seen in the graph on days 5, 7, and 9. The treatment group experienced a decrease in lymphocytes. Its content, in the form of flavonoids (as antibacterial), tannins, and saponins (anti-inflammatory), can suppress bacterial growth rates and improve wound healing.[19.21] The high concentration influenced the antibacterial activity of banana stem extract.[16]

Figure 3 and figure 4 show the high number of fibroblast cells on day 1, although its decrease followed it on the following days in the positive control group and the negative control group. The treatment group found an increase in the number from day 1 to day 3, a decrease on day 5, and an increase on day 7 to day 9. The increase in fibroblasts on day 3 in the treatment group could be caused by the slow healing process from the induction of gingivitis by bacteria. This is in line with research by Budi et al. (2019) on the inflammatory phase, from the beginning of the wound formation: until the fifth day. Existence Antigens used in wound healing can cause abnormalities in wound healing.[10] Thus, not many fibroblasts had moved to the wound area on day 1. The gingiva had undergone an inflammatory phase from day 1 to day 5 in the positive control group, marked by a decrease in fibroblasts, indicating that the healing process is nearing the final stage, followed by a slow continuous cycle replacement of collagen fibers by fibroblasts.[10] The decrease in the number of fibroblasts in the positive control group was caused by the inactive nature of fibroblasts when there was no inflammation in the patient's body, and the remaining fibroblasts only needed to change the arrangement of the collagen fibers to become more organized. The small number of fibroblast cells that appeared in the negative control group could be due to no stimulation of healing from antibacterial substances as in the other groups, so the number of fibroblasts that appeared was not as many as in the other groups.[7]

Chlorhexidine gel used as a positive control contained 0.2% chlorhexidine digluconate, with a fairly high antibacterial power. It occurred due to the bactericidal properties of antibacterial substances when the concentration was above 0.12%.[22] These bactericidal properties disrupted the stability of the cell wall and interfered with bacterial osmosis. Once the cell wall was damaged, chlorhexidine crossed into the cell and attacked the cytoplasmic membrane (inner membrane). Damage to the delicate semipermeable cytoplasmic membrane allowed leakage of components leading to cell death.[11]

The treatment group experienced slower healing due to the low content of antibacterial substances contained in the pseudostem extract gel of Ambon banana with a concentration of 20%. The pseudostem extract gel of Ambon banana contains 3.11% alkaloids, 2.36% saponins, 2.05% tannins, 0.82% flavonoids, and 0.51% anthraquinones. Some of these substances are bactericidal, although others are still bacteriostatic.[11]

This study found that the negative control group experienced an increase in the number of fibroblasts, as did the treatment and positive control groups. The negative control group used a gel base made with CMS (gelling agent), propylene glycol, glycerin, and distilled water. Some of the ingredients used have antibacterial properties in certain concentrations.[23] Treatment of Sprague-Dawley rat gingivitis in this study will still occur when applied with a gel base with or without Ambon banana pseudostem extract, which acts as a catalyst for tissue response, due to the glycerin content in the gel base as a negative control, which has a tissue healing effect. The use of glycerin on wound tissue has the effect of preventing infection in wounds because glycerin functions to reduce the number of microbes in wound tissue and shorten the time for the inflammatory process to occur so that the healing process is better.[23] Therefore, the application of pseudostem extract gel may have potential benefits for improving the healing process of gingivitis based on these findings, but it still requires further research before its clinical application.

The limitation of this research is that the choice of material as a negative control was not optimal because it still used glycerin as a negative control. Further research needs to be carried out with a negative control using distilled water so that more meaningful differences can be seen between the treatment and the negative control. Further research needs to be carried out in collaboration with pharmaceuticals to make a topical application gel for gingivitis so that the benefits of Ambon banana pseudostem extract gel as an alternative ingredient to chlorhexidine can be applied to humans.

CONCLUSION

The pseudostem extract gel of Ambon banana applied to the Sprague-Dawley group reduced the number of lymphocyte cells and increased fibroblasts in the treatment of gingivitis.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of the Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta with the number: 020/EC-KEPK FKIK UMY/III2021.

Informed Consent Statement: "Not applicable" for studies not involving humans.

Data Availability Statement: data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study

Conflicts of Interest: The authors declare no conflict of interest

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